

## FOOD DERIVED PEPTIDES STIMULATE MUCIN SECRETION AND GENE EXPRESSION IN INTESTINAL CELLS

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### Abstract

In this study, the hypothesis that food-derived opioid peptides besides  $\beta$ -casomorphin 7 might modulate the production of mucin via a direct action on epithelial goblet cells was investigated in HT29-MTX cells used as a model of human colonic surface. Seven milk whey or casein peptides, a human milk peptide and a wheat gluten-derived peptide with proved or probable ability to bind  $\mu$  or  $\delta$  opioid receptors were tested on the cell culture. Significantly increased secretion of mucins was found after 2, 4 or 8 hours of exposure to six peptides, besides the previously described  $\beta$ -casomorphin 7, as measured by an enzyme linked lectin assay (ELLA). Human  $\beta$ -casomorphin-5 and  $\alpha$ -lactorfin were selected to study the expression of MUC5AC, the HT29-MTX major secreted mucin gene, between 10 minutes up to 24 hours time of exposure.  $\alpha$ -lactorfin showed increased expression of MUC5AC at 24 hours (1.6-fold basal level expression) whereas human  $\beta$ -casomorphin-5 did not, although it tended to time-dependently increase MUC5AC expression. In conclusion, we have identified seven food-derived peptides with described or probable opioid activity that induce mucin secretion in HT29-MTX cells. Concretely,  $\alpha$ -lactorfin is able to up-regulate the expression of the main mucin gene encoded by these cells.

## INTRODUCTION

The gastrointestinal lumen is covered by a viscous solution, known as mucus, which lubricates the epithelia, helping in the passage of substances and particles through the digestive tract, and forms a protective layer against noxious chemicals, microbial infections, dehydration, and changing luminal conditions (Pérez-Vilar, 2009). The intestinal mucus gel owes its properties to secreted mucins, which are high-molecular-weight glycoproteins produced by goblet cells of the epithelium. Not surprisingly, mucin gene expression, biosynthesis and secretion are highly regulated. Disruption of the barrier integrity and invasion of microbes with subsequent chronic inflammation and further disturbance of the mucosal architecture are hallmarks of inflammatory bowel disease such as Crohn's disease and ulcerative colitis (Otte et al., 2008). Even colon cancer has been linked to a faulty mucin expression in rat model experiments (Velcich et al, 2002).

Certain dietary components such as fiber, short chain fatty acids and probiotics can positively influence characteristics of the intestinal mucus, although the mode of action of each compound may differ. Oat bran, rye bran and soybean hull were shown to increase goblet cell volume density in the proximal and distal small intestine of hamsters (Lundin et al., 1993). Among three main short-chain fatty acids produced in the human colon (i.e., acetate, propionate and butyrate), butyrate appears to be the most effective in stimulate mucus release (Shimotoyodome, 2000). Its modulation of mucin (MUC) gene expression in intestinal epithelial goblet cells has been subsequently demonstrated (Gaudier et al., 2004). Besides, the mechanisms that regulate butyrate-mediated effects on MUC2 synthesis have been studied (Burger van Paassen et al., 2009). Recently, it has been reported that selected probiotics can induce MUC3 expression of mucosal intestinal epithelial cells in a reproducible although time-limited manner (Dykstra et al., 2011).

Regarding dietary proteins, no information about their impact was available until two milk protein hydrolysates (casein and lactalbumin hydrolysates) and the peptide  $\beta$ -casomorphin 7, specifically, were shown to induce a strong release of mucin in the jejunum of the rat through the activation of the enteric nervous system and opioid receptors (Claustre et al., 2002). Trompette et al. (2003) provided evidence that peptides that had shown this effect need to be absorbed and participate in the regulation of intestinal mucus discharge through activation of  $\mu$ -opioid receptors on intestinal cells. The presence of opioid receptors on these cells suggests the possibility that food-derived peptides with opioid agonist structure, which can be produced in the intestinal lumen during gastrointestinal digestion,

might modulate the production of mucin via a direct action on epithelial goblet cells. Rat and human mucus-secreting cell lines can be used as a model to avoid interactions with the nervous system. In rat DHE cells,  $\beta$ -casomorphin 7 has shown to increase mucin secretion and the expression of rat mucin rMuc2 and rMuc3. In human HT29-MTX cells, this peptide increased as well mucin secretion and MUC5AC mRNA levels (Zoghbi et al 2006). The aim of this work was to evaluate if other food peptides, besides  $\beta$ -casomorphin 7, can induce mucin secretion and MUC5AC expression on human HT29-MTX intestinal cells.

## **MATERIALS AND METHODS**

### **Samples**

$\beta$ -casomorphin-7 and (D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,glycinol<sup>5</sup>)enkephalin (DAMGO) were purchased from Bachem (Bubendorf, Switzerland). Human neocasomorphin (YPVEPF), human  $\beta$ -casomorphin 5 (YPFVE),  $\alpha$ -casein exorphin (YLGYLE), bovine  $\beta$ -lactorphin (YLLF-NH<sub>2</sub>), human and bovine  $\alpha$ -lactorphin (YGLF-NH<sub>2</sub>), gluten exorphin A5 (GYIPT) and the  $\alpha$ -casein fragments 90-94 (RYLGY) and 143-149 (AYFYPEL) were synthesized using conventional solid-phase Fmoc synthesis with a 433A peptide synthesizer (Applied Biosystems). Their purity (>90%) was verified in our laboratory by reverse phase high performance liquid chromatography and tandem mass spectrometry.

### **Cell culture**

HT29-MTX, a human colon adenocarcinoma-derived mucin-secreting goblet cell line was provided by Dr. Thécla Lesuffleur (INSERM UMR S 938, Paris, France) (Lessuffleur et al. 1993). The cell line was grown in plastic 75-cm<sup>2</sup> culture flasks in DMEM supplemented with 10% FBS and 100 mg/ml penicillin-streptomycin (all from Invitrogen) at 37<sup>o</sup> C in a 5% CO<sub>2</sub> atmosphere in a humidified incubator. Cells were passaged weekly using trypsin/EDTA 0.05% (Invitrogen) in Ca<sup>2+</sup> - Mg<sup>2+</sup> -free PBS. The culture medium was changed every two days. To study the effect of peptides or DAMGO, cells were seeded at a density of 5×10<sup>5</sup> cells per well in 12-well culture plates (Nunc). The cell line was used between passages 12 and 19. Experiments were performed 21 days after confluency. Twenty-four hours before the studies, the culture medium was replaced by serum- and antibiotic-free medium to starve the cells and to eliminate any interference from extraneous proteins or hormones. After serum-free medium removal, the monolayer was washed twice with PBS. Serum-free medium with or without peptide

(0.05-0.5mM) or DAMGO (0.001mM) was added to the cells and incubated at 37°C for 10 min to 24 hours in a 5% CO<sub>2</sub> humidified atmosphere. The supernatants were collected, frozen and stored at -70°C. The total RNA was isolated with Nucleospin® RNA II (Macherey-Nagel).

### **Enzyme-Linked Lectin Assay**

To measure mucinlike glycoprotein secretion an enzyme-linked lectin assay (ELLA) reported by Trompette et al. (2000), slightly modified, was used. Briefly, wells of a microtiter plate were coated with sample diluted in sodium carbonate buffer (0.5M, pH 9.6) and incubated overnight at 4°C. The plates were then washed with PBS containing 0.1% Tween (PBS-Tween, pH 7) and blocked with 2% BSA in PBS-Tween for 1h at 37°C. After samples were washed six times, biotinylated wheat germ agglutinin (1:1000) in PBS-Tween-BSA was added, and the samples were incubated for 1 h at 37° C. Subsequently, avidin-peroxidase conjugate (1:50000) was added for signal amplification. Colorimetric determination using o-phenylenediamine dihydrochloride solution (Dako) was performed at 492 nm. Mucinlike glycoprotein content of samples was determined from standard curves prepared from gastric porcine mucin (Sigma). All experiments were performed in triplicate.

### **Real-time quantitative RT-PCR assays (qRT-PCR)**

Quantitative RT-PCR amplification was carried out with the real-time fluorescence method using a 7500 Fast System (Applied Biosystems). RNA (375 ng) was reverse transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to the manufacturer's instruction. The specific primers (target and reference genes) used for the RT-PCR assays are listed in **Table 1**. The SYBR Green method was used and each assay was performed with cDNA samples in triplicate. Each reaction tube contained 12,5 µL 2x SYBR Green real-time PCR Master Mix (Applied Biosystems), 5µL of a 1µM of gene-specific forward and reverse primers, and 2,5 µL of a 1:10 dilution of cDNA. Amplification was initiated at 50°C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Control PCRs were included to confirm the absence of primer dimer formation (no-template control), and to verify that there was no DNA contamination (without RT enzyme negative control). All real-time PCR assays amplified a single product as determined by melting curve analysis and by electrophoresis in 2% agarose gels. A standard curve was plotted with

cycle threshold (Ct) values obtained from amplification of known quantities of cDNAs and used to determine the efficiency (E) as  $E = 10^{-1/\text{slope}}$ .

The relative expression levels of the target gene were calculated using the comparative critical threshold method ( $\Delta\Delta\text{Ct}$ ). Human cyclophilin and  $\beta$ -actin were assayed as reference genes. Cyclophilin gene was chosen to calculate the threshold cycles because it had previously been shown to be constant under all conditions used here (data not shown). All experiments were performed at least three times in triplicate.

### **Statistical analysis**

Data were analyzed by a two-way ANOVA, using the GraphPad Prism 4 software, followed by the Bonferroni test for single comparisons. Differences between means and controls were considered significant with  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) or  $p < 0.001$  (\*\*\*) .

## **RESULTS**

### **Determination of the mucin secretion of HT29-MTX cell culture over 24 hours**

HT29-MTX cells form a homogeneous monolayer of polarized goblet cells that exhibit a discrete apical brush border (Lessuffleur et al., 1990). Previous studies have shown that the morphological differentiation of the cells, as well as the secretion of mucins when it occurs, is a growth-related phenomenon, starting after the cells have reached confluency (Lessuffleur et al., 1993). In order to get a quantitative information about the mucin production by HT29-MTX human colonic cells during 24 hours, mucins were quantified by ELLA when the proportion of cells that express mucus reaches 100% and remains stable, which had been reported to be 21 days (Lessuffleur et al., 1993). **Fig. 1** shows the concentration of mucin-like glycoproteins found in the culture medium at increasing times between 30 min and 24 hours. The values exhibited a steep increased secretion of mucin between 4 and 8 hours (ten times higher) that was followed by a further increase reaching six times the 8 hours-value at 24 hours. Very close values were measured in two independent experiments for each time. Therefore, the cell culture proved to be a reliable tool for the study of gastrointestinal mucin secretion.

### **Mucin secretion of HT29-MTX cells under the effect of different food peptides**

Various synthetic milk or wheat-derived gluten peptides with proved ability to bind  $\mu$  or  $\delta$  opioid receptors and two casein-derived peptides that had shown a potent antihypertensive effect and whose sequence may anticipate opioid activity were added to the cell culture (Contreras et al., 2009). The specific  $\mu$ -receptor agonist DAMGO was used as a positive control. Fig. 2 shows time-course experiments of addition of 0.1 mM of peptide, the optimal concentration for  $\beta$ -casomorphin previously described (Zoghbi et al, 2006) and subsequent determination of secreted mucin by ELLA. Peptide  $\alpha$ -lactorphin (YGLF-NH<sub>2</sub>), which derives from both human and bovine  $\alpha$ -lactalbumin showed the highest mucin secretion level after 4 hours, although the result was also significantly high at 2 hours, which denotes the enhanced mucus discharge in this time range (Fig. 2 A). Human  $\beta$ -casomorphin 5 (YPFVE) showed increased values of mucin secretion at 0.5 and 2 hours followed by a highly significant increase at 4 hours, and a less significant value at 8 hours (Fig. 2 B). Peptides from bovine  $\alpha$ <sub>s1</sub>-casein RYLGY and AYFYPEL showed lower absolute although significant values after 2 and 4 hours, respectively (Fig. 2 C, D).

**Table 2** summarizes the maximum mucin secretion of HT29-MTX cells after addition of the assayed peptides. Six from nine studied food peptides showed highly significant ( $P < 0.001$ ) activity on mucin secretion by HT29-MTX. Gluten exorphin showed a less activity ( $p < 0.05$ ). Secretion values ranged from 157.2 to 587.3 % respect to control. Significant increases took place mainly after 4 hours exposure but peptide RYLGY showed maximum mucin secretion after 2 hours. The specific  $\mu$ -receptor agonist DAMGO behaved as a potent mucus secretagogue, in HT29-MTX cells, this activity having been previously found in rat *ex-vivo* experiments (Trompette et al., 2003) and DHE cells (Zoghbi et al., 2006).

### **MUC5AC expression in HT29-MTX cells under the effect of different food peptides**

Quantitative PCR was used to determine the level of transcripts of MUC5AC in HT29-MTX cells treated with  $\alpha$ -lactorphin (YGLF-NH<sub>2</sub>) and human  $\beta$ -casomorphin-5 (YPFVE) peptides, as the whey and casein peptides where highest mucin secretion had been observed, respectively. MUC5AC was selected because it is one of the genes which is expressed concomitantly with the secreted mucins of gastric-like phenotype and presents the highest levels of mRNA in HT29-MTX cells.  $\beta$ -casomorphin 7 was used as positive control and it showed increased expression levels of MUC5AC after 24 hours of exposure (1.7-fold basal level), according to the previously reported results (Zoghbi et al. 2006).

Increasing concentrations of peptides between 0.05 and 0.5 mM were added to the incubation medium of HT29-MTX cells at times in the range 10 minutes to 24 hours (Fig. 3).  $\alpha$ -lactorphin elicited significantly ( $P < 0.05$ ) increased transcripts of MUC5AC at 24 hours (1.6-fold basal level expression) when the highest dose, 0.5 mM, was added. Moreover, further additional experiments with this peptide have shown induced expression (1.3-fold basal level) at 8 and 18 hours exposure (results not shown). On the contrary, YPFVE showed levels of MUC5AC not significantly different than the control, although this peptide tended to time-dependently increase MUC5AC expression.

## DISCUSSION

Mucin secretion by HT29-MTX goblet cells increased noticeably along the studied period of 24 hours. The slow rise between 0.5 and 4 hours may be related to cells adaptation after being submitted to similar conditions as those of the assay. The observed trend is in accordance with information provided in the literature about the high mucin-producing capacity by intestinal goblet cells, based on the important role that mucins play in the epithelium protection and lubrication, as well as its constant renewal due to its elimination by the intestinal peristalsis and continuous secretion (Linden et al, 2008).

$\beta$ -casomorphin 7 was the first food peptide reported with opioid activity (Brantl et al 1979). It is the most studied food-derived opioid peptide and its influence on the mucin secretion has been evaluated *in vitro* (human and rat) and *ex vivo* (rat) (Trompette et al. 2003; Zoghbi et al., 2006). Its activity on the secretion of mucin and MUC5AC expression by goblet cells is confirmed by our results. Besides, a whey protein-derived peptide,  $\alpha$ -lactorfin, with proved opioid activity, although with lower affinity towards  $\mu$  receptors than  $\beta$ -casomorphin 7 (Yoshikawa et al., 1986), has been found to induce mucin secretion and MUC5AC gene expression. Our results have not permitted to find a relationship of the effect of peptide-induced gene modulation to dose, because significance on level of transcripts of MUC5AC was only found at the highest dose assayed (0.5mM). Nevertheless, it has to be considered the thickness of the mucus layer in our cell model and the passage of peptide to reach the opioid receptors. Regarding the time for MUC5AC expression, the maximum value was always found after 8 to 24 hours exposure. The mucin discharge coupled with the corresponding increase of MUC gene expression to replenish the intracellular mucin pool is a behaviour that can be found in other secreting cells like pancreatic  $\beta$  cells, which respond to changes in blood glucose by first secreting insulin and

next increasing insulin biosynthesis (Webb et al. 2000). The time range where stimulation of glycoprotein synthesis and mucin exocytosis reach their maximum under the effect of external agents is still unknown. A study on treatment of rat cells with butyrate showed that the significant increase in MUC gene expression was observed after 24 hours but not at the earlier time points tested (1, 3 and 8 hours) (Gaudier et al. 2004).

Human  $\beta$ -casomorphin-5 showed a significant mucin secretory activity but no over expression of MUC5AC was found in the analysed samples. Human beta-casomorphin-5 displays opioid activity with affinity for  $\mu$  and  $\delta$  receptors although it is 2.6 times less potent than  $\beta$ -casomofin 7 (Koch et al. 1985). However, given the significant values shown on mucin secretion, even though the behaviour was less acute than that of  $\alpha$ -lactorfin, it would be of interest to analyse the expression of MUC5AC using additional times of exposure to this peptide, as increased expression could likely be found as longer exposure times.

Two peptides from bovine  $\alpha$ -casein, RYLGY and AYFYPEL, showed significant mucin-secretor values. These sequences have not been reported as opioid but have been described in a hydrolysate prepared by our research group where antihypertensive activity has been demonstrated (Contreras et al., 2009). Peptide RYLGY is included into the sequence of a casein exorphin (RYLGYL) with moderate opioid activity and  $\mu$  and  $\delta$  receptors affinity (Loukas et al., 1983). In this case, it would be interesting to evaluate if the absence of the Leu residue might improve interaction with opioid receptors. Moreover, the mucin-secretory activity of RYLGY, gets maximum at 2 hours. This would mean a faster activity for this peptide on cells and could be determinant if conclusions about the gastrointestinal flow should be taken trying to reach a fast response upon ingestion. Peptide AYFYPEL had not been previously described as opioid peptide but shows an aromatic residue Tyr in the second position and Phe together with Tyr in third and fourth positions, respectively, a favourable structure to bind opioid receptors (Merke et al., 1990).

Gluten exorphin A5, a peptide whose opioid activity had been demonstrated (Fukudome et al., 1997) presented mucin secretion activity. The low value encountered is in accordance with the lower mu-receptor affinity of this peptide compared to  $\delta$  receptor (Fukudome et al., 1997). Even so, this is the first report of the mucin-secretory activity of this opioid peptide of vegetal origin on human HT29-MTX goblet cells.

Finally, peptides showing no stimulatory activity, neocasomorphin and  $\alpha$ -casein exorphin 2-7 (YLGYLE), although having been previously reported as opioid peptides, have been shown lower activity affinity for  $\mu$  and  $\delta$  receptors than  $\beta$ -casomorphin-7 (Jimsaa and Yoshikawa, 1999). However, this lower affinity cannot explain the lack of activity found for these peptides, since the affinity of neocasomorphin for  $\mu$  receptors is higher than that of  $\alpha$ -lactorphin.

The fact that not only casein-derived but also whey-derived peptides provoke a mucin-expression modulation opens a new sight, regarding to previous works, where  $\beta$ -casomorphins were solely considered to play an important physiological role in the gastrointestinal tract. In fact, casein has demonstrated *in vivo* an effect on intestinal mucin expression in the rat, where a significant increase of Muc3 mRNA in the small intestinal tissue and Muc4 mRNA in the colon has been observed when a diet containing hydrolysed casein compared to a synthetic amino acid mixture or a protein-free diet was orally administered (Han et al, 2008). There have been studies related the administration of bovine  $\alpha$ -lactalbumin and the stimulation of mucus metabolism in gastric mucosa (Ushida et al., 2003; 2007) and some reports had evidenced the activity of  $\alpha$ -lactalbumin and hydrolysates of this protein on gastric ulcer on rat models *in vivo* (Mezzaroba et al., 2006). Further, a hydrolysate of this protein induced a strong release of mucin in the jejunum of the rat *ex-vivo* (Claustre et al., 2002). However, some researchers support the view that the protection of whey protein on induced colitis in rats has to be attributed to its high content in threonine and cysteine and to a reduced gene expression of inflammation markers such as interleukin 1 $\beta$ , calprotectin and inducible nitric oxide synthase (Sprong et al., 2010). Indubitably, the effect of dietary peptides on the mucus protection mechanisms needs to be ascertained.

In conclusion, seven food-derived peptides have shown to induce mucin secretion in HT29-MTX human colonic goblet like cells for the first time. Some of them had been previously described as opioid peptides but two sequences had not, although their structure may be favourable to bind opioid receptors. Concretely  $\alpha$ -lactorphin, which is found both in human and bovine milk, increased the expression of MUC5AC. This is a first step in finding new food peptides that can be included in the wide variety of stimuli that provoke mucin secretion in goblet cells and therefore play a role in the modulation of this protective function. These findings may assist in the development of dietary strategies to augment mucus layer formation as protection against inflammatory bowel disease effects.

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## FIGURE CAPTIONS

**Figure 1.** Time –course secretion of mucin in HT29-MTX cells determined by enzyme-linked lectin assay (ELLA). Data are expressed as mucin-like glycoprotein secretion. Each point represents the mean  $\pm$  SE (n=6)

**Figure 2.** Time-course effect of YGLF-NH2 ( $\alpha$ -lactorphin) (A), YPFVE (human  $\beta$ -casomorphin) (B), RYLGY (C) and AYFYPEL (D) on mucin secretion in HT29-MTX cells determined by enzyme-linked lectin assay (ELLA). Data are expressed as mucinlike glycoprotein secretion as a percentage of control. Each point represents the mean  $\pm$  SE (n=3). Significant differences by two-way ANOVA applying the Bonferroni test: \*\*\*p<0.001; \*p<0.05

**Figure 3.** Effect of YGLF- NH2 (A) and YPFVE (B) on MUC5AC mRNA level determined by real-time quantitative RT-PCR (qRT-PCR) after 10 min to 24 h of exposure by using  $\beta$ -actin as control. Each point represents the mean of three independent biological replicates  $\pm$  SE (n=3). Significant differences by two-way ANOVA applying the Bonferroni test: \*p<0.05

Table 1. Primers for real-time PCR

Gene	Amplicon size (pb)	Primers	Reference
MUC5AC	240	5'- CGACCTGTGCTGTGTACCAT-3'	(2870-2889)
		5'- CCACCTCGGTGTAGCTGAA-3'	(3109-3091)
Human cyclophilin	165	5'- TCCTAAAGCATACGGGTCCTGGCAT-3'	(280-304)
		5'- CGCTCCATGGCCTCCACAATATTCA-3'	(445-421)
Human beta actin	197	5'- CTTCTGGGCATGGAGTC-3'	(879-896)
		5'- GCAATGATCTTGATCTTCATTGTG-3'	(1076-1053)

Table 2. Maximum mucin secretion in HT29-MTX cells upon addition of food peptides determined by enzyme-linked lectin assay (ELLA). Significant differences between average values (n=3) and control by two-way ANOVA (Bonferroni test).

Sequence	Peptide		Time (h)	Secreted mucin	
	Denomination	Food protein		% Control	P
YPFPGPI	Bovine $\beta$ -casomorphin 7	$\beta$ -casein A2 f(60-66)	4	282	<0.001
YPVEPF	Neocasomorphin	$\beta$ -casein f(114-119)	-	-	-
YPFVE	Human $\beta$ -casomorphin	$\beta$ -casein f(51-55)	4	339.2	<0.001
YLGYLE	$\alpha$ -casein exorphin	$\alpha$ -casein f(91-96)	-	-	-
RYLGY	-	$\alpha$ -casein f(90-94)	2	191.3	<0.001
AYFYPEL	-	$\alpha$ -casein f(143-149)	4	220.8	<0.001
YLLF-NH <sub>2</sub>	$\beta$ -lactorphin	$\beta$ -lactoglobulin f(102-105)	4	452.9	<0.001
YGLF-NH <sub>2</sub>	$\alpha$ -lactorphin	$\alpha$ -lactalbumin f(50-53)	4	587.3	<0.001
GYYPYPT	Gluten exorphin A5	Wheat glutenin	4	157.2	<0.05
-	DAMGO		4	232.3	<0.01





